ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2011, p. 4238–4250 Vol. 55, No. 9
0066-4804/11/$12.00 doi:10.1128/AAC.00267-11


# Humanized θ-Defensins (Retrocyclins) Enhance Macrophage Performance and Protect Mice from Experimental Anthrax Infections▽

S. Welkos,[1]*† C. K. Cote,[1]† U. Hahn,[2] O. Shastak,[2] J. Jedermann,[2] J. Bozue,[1] G. Jung,[3] P. Ruchala,[3] P. Pratikhya,[3] T. Tang,[3] R. I. Lehrer,[3]*† and W. Beyer[2]†

*Bacteriology Division, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland[1]; University of Hohenheim, Stuttgart, Germany[2]; and David Geffen School of Medicine at UCLA, Los Angeles, California[3]*

Received 25 February 2011/Returned for modification 17 June 2011/Accepted 3 July 2011

**Retrocyclins are humanized versions of the θ-defensin peptides expressed by the leukocytes of several nonhuman primates. Previous studies, performed in serum-free media, determined that retrocyclins 1 (RC1) and RC2 could prevent successful germination of *Bacillus anthracis* spores, kill vegetative *B. anthracis* cells, and inactivate anthrax lethal factor. We now report that retrocyclins are extensively bound by components of native mouse, human, and fetal calf sera, that heat-inactivated sera show greatly enhanced retrocyclin binding, and that native and (especially) heat-inactivated sera greatly reduce the direct activities of retrocyclins against spores and vegetative cells of *B. anthracis*. Nevertheless, we also found that retrocyclins protected mice challenged *in vivo* by subcutaneous, intraperitoneal, or intranasal instillation of *B. anthracis* spores. Retrocyclin 1 bound extensively to *B. anthracis* spores and enhanced their phagocytosis and killing by murine RAW264.7 cells. Based on the assumption that spore-bound RC1 enters phagosomes by "piggyback phagocytosis," model calculations showed that the intraphagosomal concentration of RC1 would greatly exceed its extracellular concentration. Murine alveolar macrophages took up fluorescently labeled retrocyclin, suggesting that macrophages may also acquire extracellular RC1 directly. Overall, these data demonstrate that retrocyclins are effective *in vivo* against experimental murine anthrax infections and suggest that enhanced macrophage function contributes to this property.**

The infectious form of *Bacillus anthracis* is the dormant spore. To cause anthrax, this spore must germinate, grow within the host, form a capsule, and release various exotoxins, which include lethal toxin, edema toxin, and anthrolysin (18, 23, 39, 47). Without effective treatment, *B. anthracis* can proliferate and cause death. Agents that neutralize *B. anthracis* toxins, permanently prevent germination of *B. anthracis* spores, and/or kill or control vegetative *B. anthracis* growth could protect against this natural pathogen and class A bioterrorism agent.

Defensins and defensin-like peptides are components of the innate immune system. (21, 31). Three defensin subfamilies, designated α, β, and θ, exist among mammals. Three α-defensins, called human neutrophil peptides (HNPs) 1, 2, and 3, collectively constitute 5 to 7% of the total protein of human neutrophils (polymorphonuclear cells [PMN]) (33) and can kill *B. anthracis* bacilli, inactivate anthrax lethal toxin *in vitro*, and protect mice injected with anthrax lethal toxin *in vivo* (27). The same α-defensins also play an important role in the potent anti-*B. anthracis* activity of human neutrophils (37). Retrocyclins, the synthetic peptides examined in this study, are humanized analogs of the θ-defensin peptides found in the leukocytes of rhesus macaques and other nonhuman primates (22, 45). θ-Defensin genes arose by mutation of α-defensin genes (22, 40, 45). Human θ-defensin genes exist and are transcribed, but the human genes and transcripts contain a premature stop codon that aborts translation (8), unless the cells are induced to read through the stop codon (49). Curiously, whereas rat and human PMN contain multiple α-defensins, murine PMN contain none (15, 40).

Retrocyclins inactivate anthrax lethal toxin and kill *B. anthracis* bacilli *in vitro* (51), and they are more effective than HNP 1 to 3 in preventing *B. anthracis* spores from germinating and commencing vegetative growth (51). Based on these findings, we performed additional *in vitro* studies with retrocyclins and *B. anthracis* and tested their ability to protect mice challenged *in vivo* with *B. anthracis* spores.

## MATERIALS AND METHODS

**Retrocyclins.** Retrocyclin 1 (RC1) and RC2, >95% pure, were synthesized at UCLA as previously described (8). To obtain a fluorescent retrocyclin analog, we made a precursor whose linear sequence (GICRCICGRKICRCICGR) was identical to RC1, except for the underlined arginine-to-lysine substitution. We used the free ε-amino group of the introduced lysine to covalently attach a fluorescent dye, Alexa Fluor 568 (Invitrogen, Carlsbad, CA). To minimize spatial overlap between the Alexa Fluor 568 and the peptide, we first coupled a flexible, hydrophilic polyethylene glycol spacer (catalog number 01-63-0200; EMD Biosciences, San Diego, CA) to this ε-amino group. Attachment of the spacer and Alexa Fluor 568 were done stepwise in dimethyl sulfoxide, using *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (10 equivalents) plus *N*-hydroxysuccinimide (50 equivalents) and *N*-methylmorpholine (25 equivalents). The final product was purified to >95% homogeneity by reverse-phase high-performance liquid chromatogra-

* Corresponding author. Mailing address for R. I. Lehrer: David Geffen School of Medicine, UCLA, 10833 Le Conte Ave., Los Angeles, CA 90095. Phone: (310) 825-5340. Fax: (310) 206-8766. E-mail: rlehrer@mednet.ucla.edu. Mailing address for S. Welkos: Bacteriology Division, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD 21702. Phone: (301) 619-4930. Fax: (301) 619-2152. E-mail: susan.welkos@amedd.army.mil.

† S. Welkos, C. K. Cote, R. I. Lehrer, and W. Beyer contributed equally to this publication.

▽ Published ahead of print on 18 July 2011.

phy and lyophilized. The retrocyclin moiety constituted approximately 2/3 of its mass. Protegrin 1 is a noncyclic, potently antimicrobial peptide that was originally isolated from porcine leukocytes (28). Like retrocyclins, protegrin 1 (PG1) contains 18 amino acids and has two antiparallel β-sheets that are bridged by disulfide bonds. Unlike retrocyclins, PG1 is noncyclic and contains only 4 cysteines. The synthetic HNP1 used in binding and radial diffusion assays was the generous gift of Wuyuan Lu at the Institute for Human Virology of the University of Maryland.

**Serum.** Mouse serum from Jackson Laboratories (Bar Harbor, ME), PromoCell (Heidelberg, Germany), or Gemini Bio-Products (Woodland, CA) was stored at −80°C until used. Fetal bovine serum (FBS) was from Invitrogen or Gemini Bio-Products. Human serum was from PromoCell (Heidelberg, Germany), Innovative Research (Sarasota, FL), or Gemini Bio-Products (Woodland, CA). In some experiments, "careful" human serum was prepared from fresh human blood as previously described to minimize its content of HNP 1 to 3 (41).

**Studies with LF.** Recombinant lethal factor (LF) and anthrax LF protease substrate III were purchased from Calbiochem. The substrate (Ac-Gly-Tyr-β-Ala-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Val-Leu-Arg-C-7-amido-4-methylcoumarin) simulates a MEK-2 template. Enzyme assays were done as previously described (51).

***B. anthracis* spores.** Spores were obtained from three *B. anthracis* strains: fully virulent Ames strain; ΔAmes, an Ames derivative lacking the pX01 toxin-encoding plasmid (26), and the toxigenic but nonencapsulated Sterne strain. Tryptone glucose broth, pH 7.2, contained, per liter, 2.5 g yeast extract, 5.0 g tryptone, and 1.0 g glucose. Meat yeast agar, pH 7.0, contained, per liter, 10 g meat extract, 2 g yeast extract, 40 mg $MnSO_4 \cdot H_2O$, and 15 g agar. In Stuttgart, tryptone glucose broth was inoculated with $10^6$ spores and incubated at 30°C. When the culture contained $10^7$ CFU/ml, 2.5 ml was transferred to a bottle containing 30 ml of sterile meat yeast agar and gently rotated to cover the agar surface with the inoculum. After incubation at 30°C for 3 days, sporulation was evaluated regularly by phase-contrast microscopy. The culture was further incubated, for up to 10 days, until vegetative cells almost completely disappeared. Spores, recovered by flushing the agar with distilled $H_2O$, were centrifuged at 3,000 × *g* for 10 min. The pellet was resuspended in 65% isopropanol for 2 to 3 h at room temperature to inactivate vegetative bacilli. The suspension was diluted with $H_2O$ and centrifuged again. The spore pellet was washed five times with sterile physiological saline that contained 0.1% (wt/vol) gelatin to prevent clumping. After the final wash, spores were resuspended at the desired concentration and stored at 4°C. Spore numbers and purity were determined by plating appropriate dilutions on blood agar and verified by microscopy after staining. The number of remaining vegetative bacilli was assessed by microscopy after staining. Before the spore suspensions were used in challenge experiments, they were heated at 65°C for 30 min to ensure that dormant spores were the only viable form used.

At USAMRIID, spores of *B. anthracis* Ames were obtained by culturing the organisms in Leighton-Doi sporulation medium (35). After the spores were thrice washed with water, they were centrifuged through Hypaque-76 (Nycomed, Inc., Princeton, NJ) and washed three more times (11). Before use, the spore suspensions were heated at 65°C for 30 min and then held on ice (11). At UCLA, *B. anthracis* Sterne spores were prepared as previously described (3) for use in the binding and kinetic growth assays described below.

**Spore germination and bactericidal testing.** Vegetative cells were prepared from stock *B. anthracis* spore suspensions for *in vitro* studies. Nonencapsulated bacilli were prepared by germinating $1 \times 10^6$ to $2 \times 10^6$ spores at 37°C in 1 ml of either human, mouse, or fetal calf serum for 90 min, by which time >99.99% of spores had germinated. When encapsulation was desired, incubation took place in a 20% $CO_2$ atmosphere. Treatments in human or mouse serum were done using 100 μl of the suspension, right after the germination of spores. For treatment in buffer, the cells germinated in fetal calf serum were centrifuged at 13,000 × *g* for 2 min and the pellet was resuspended in 1 ml of buffer, usually phosphate-buffered saline (PBS; 8 g/liter NaCl, 0.2 g/liter KCl, 0.91 g/liter $Na_2HPO_4 \cdot 2H_2O$, 0.12 g/liter $KH_2PO_4$) plus 1% (vol/vol) brain heart infusion broth. Mixtures containing $1 \times 10^5$ freshly germinated *B. anthracis* cells and various retrocyclin concentrations were incubated at 37°C for 2 h. Buffer-only controls were included to detect spontaneous loss in viability during the incubation. Bactericidal activity was evaluated by comparing viable counts of retrocyclin-treated and nontreated controls, which were normalized to the cell count after germination.

**Kinetic assessment of *B. anthracis* growth and survival.** *B. anthracis* Sterne spores were incubated overnight at 30°C in tryptic soy broth (TSB). The next morning, a subculture was initiated by transferring 1 ml of this overnight culture into 10 ml of fresh TSB, which was incubated at 30°C until its optical density (OD) at 600 nm reached 0.5. At this point, the subculture was diluted 1:10 with cold Dulbecco's minimal essential medium (DMEM) and placed on ice to stop further multiplication. For the standard curve, five additional serial 1:10 dilutions of the once-diluted subculture were prepared in DMEM and kept on ice until used. Stock solutions of RC1 were prepared in Dulbecco's minimal essential medium at twice their desired final concentrations (100, 50, 25, 12.5, 6.25. 3.12, 1.56, and 0.78 μg/ml).

Assays were done in sterile 96-well, polystyrene cell culture plates (Costar, catalog number 3596; Corning, Corning, NY). Each well contained a final volume of 100 μl, composed of Dulbecco's minimal essential medium, RC1, and 10 μl of vegetative *B. anthracis* cells. A standard curve was obtained by inoculating 100 μl of DMEM with 5 serial 10-fold dilutions of the starting *B. anthracis* cell concentration. After incubation for 90 min at 37°C, 100 μl of full-strength tryptic soy broth (Sigma, St. Louis, MO) was added to each well. Subsequent growth took place at 37°C in a UV-Vis microplate spectrophotometer (Spectra Max Gemini XS; Molecular Devices) and was monitored every 5 min at 650 nm for 16 h, with shaking by the instrument before each reading. The endpoint was the time required for each sample to increase its starting OD by 0.1 absorbance units. This value was used to obtain the standard curves and equations used to calculate the growth inhibitory effect of RC1. Additional details about this procedure can be found elsewhere (16).

**Radial diffusion assays.** In addition to measuring the intrinsic bactericidal activity of antimicrobial peptides (34, 43), this technique can also detect inhibitory substances. The assay is performed by distributing the organisms of interest, either dormant spores or vegetative-phase bacilli, throughout a thin gel of defined composition. Wells large enough to accommodate a small volume (8 μl in our studies) of serially diluted peptide solution are punched into the gel. After adding the peptides and incubating the plates at 37°C for 3 h, a rich overlay gel is poured atop the underlay and the plates are incubated overnight. The diameters of the clear zones that surround the wells are measured and used to compute the minimal effective concentration (MEC) of the peptide (34). The advantages of this approach for antimicrobial peptides, instead of conventional MIC assays, are also described elsewhere (43, 48).

**Binding of RC1 to *B. anthracis* spores.** The RC1 binding experiments were performed at UCLA, using *B. anthracis* Sterne spores, whose concentration was established by hemocytometer counting. Triplicate samples were prepared in prelubricated, 1.7-ml microcentrifuge tubes (Costar, catalog 3207; Corning). Each tube contained, in a final volume of 1 ml, Dulbecco's minimal essential medium (serum free) plus 5 μg/ml of RC1 (2.6 μM) or HNP1 (1.47 μM) with or without $8.64 \times 10^7$ *Bacillus anthracis* Sterne spores. After a 15-min incubation on ice, the tubes were centrifuged to deposit the spores. The supernatant was removed and the RC1 or HNP1 concentration was determined by surface plasmon resonance measurements performed on a BiaCore3000 instrument. The biosensor was a CM5 chip that presented gp120 from HIV-1LAV, a target that is a high-affinity ligand for RC1 (50). A standard curve was constructed by incubating various concentrations of RC1 or HNP1 in spore-free and serum-free DMEM under the same conditions.

**Binding of retrocyclin by serum.** Surface plasmon resonance studies were performed with a Biacore 3000 instrument with a CM5 biosensor to which 1,915 resonance units (RU) of gp120 from HIV-1 had been affixed by amine coupling, using the manufacturer's protocol and Biacore reagents. The analytes were prepared in Dulbecco's minimal essential medium (catalog number 11995; Gibco/Invitrogen) and contained various concentrations of retrocyclin with or without human, mouse, or fetal calf serum as described below. The flow rate was 50 μl/min, and the duration of binding was 3 min.

**Association of retrocyclin with alveolar macrophages.** Alveolar macrophages were harvested from normal uninfected BALB/c mice by bronchoalveolar lavage, using PBS containing 1% bovine serum albumin. Adherent alveolar macrophages were isolated by incubating lavage fluids in a 96-well tray for 1 h at 37°C in 5% $CO_2$. The fluorescent retrocyclin preparation, initially dissolved in 50 μl dimethyl sulfoxide, was then greatly diluted with sterile water to prepare a 0.5-mg/ml stock solution that was added to the wells to provide final RC concentrations of 0, 10, 20, and 50 μg/ml. After incubating the cultures for 3 h at 37°C in 5% $CO_2$, samples were examined with an inverted fluorescence microscope.

**Phagocytosis and killing.** Murine RAW 264.7 cell line macrophages were infected with heat-treated, ungerminated spores of *B. anthracis* Ames at a multiplicity of infection of 1 to 2 spores/macrophage, as described previously (10, 11, 54). Before adding the spores to the macrophage cultures, the spores were preincubated on ice for 30 min with 0, 5, or 50 μg/ml of RC1 in Dulbecco's minimal essential medium supplemented with 3.5 g/liter sucrose, 1% L-glutamine, and 7.5% heat-inactivated (HI) FBS (Invitrogen). When the spores reached room temperature, they were added to the macrophages. These cultures were centrifuged for 10 min at 1,150 rpm (300 × *g*) at 30°C and then incubated at 37°C in 5% $CO_2$ for 50 min. Next, the wells were extensively washed and the

medium was replaced with DMEM containing 10% heat-inactivated FBS plus 5 μg/ml of cytochalasin D to block further ingestion and 2.5 μg/ml of gentamicin to kill extracellular *B. anthracis*. Finally, after incubating the cultures for an additional 30 min at 37°C, the wells were washed again and either harvested for viable count determinations or fixed and stained as described below. Viable counts were determined on lysates of infected macrophages from three replicate wells per treatment group. The lysates, produced by a 5-min exposure to 0.1% Triton X-100, were serially diluted and plated on tryptic soy agar plates. Two replicate wells per treatment group were fixed in methanol or 5% formaldehyde (Tousimis EM grade) in PBS for 15 min. The former were air dried and stained with spore stain, and the latter were washed with PBS and blocked overnight at 4°C in PBS with 7% milk blocker. These samples were then subjected to a malachite green–Diff-Quik spore stain or fluorescent immunostaining, performed as previously described (10, 12, 54). The immunostaining used a rabbit IgG anti-*B. anthracis* prepared against germinated spores of *B. anthracis* Ames as the primary antibody and used fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit Ig from Invitrogen/Molecular Probes as secondary antibodies (10, 12). The primary antibody recognized germinated and ungerminated spores and vegetative bacilli (10, 12). Nonpermeabilized RAW 264.7 cells were incubated with the primary antibody, washed, and incubated with TRITC-labeled anti-rabbit IgG to detect extracellular spores and bacilli. Afterwards, the cells were permeabilized with 1% Triton X-100 in PBS, again incubated with the anti-*B. anthracis* IgG, and exposed to a goat anti-rabbit anitbody-FITC conjugate to stain the internalized organisms. Vectashield mounting medium (Vector Labs) containing 4′,6-diamidino-2-phenylindole was used to allow us to detect the macrophages. Additional samples were stained with malachite green, which stains ungerminated spores, and counterstained with a Wright-Giemsa stain (Diff-Quik), which stains *B. anthracis* bacilli and germinated spores but not ungerminated spores (55). Diff-Quik fails to stain germinated spores and *B. anthracis* bacilli that have been killed and degraded by macrophages (12, 54).

Neither staining procedure, when used alone, distinguishes live from dead *B. anthracis*. However, when the two stains are combined, they allow the minimal extent of killing within the phagocyte population to be estimated. This follows logically from the fact that live *B. anthracis* organisms exist in one of three forms: ungerminated spores, germinated spores, and bacilli. Since malachite green stains both germinated and ungerminated spores and Diff-Quick stains germinated spores and bacilli, use of both stains will detect all *B. anthracis* forms that may be alive. Conversely, the "in/out" stain detects the total number of *B. anthracis* that are intracellular. If one knows the total number of organisms that were ingested and the maximal number that may still be alive, one can calculate the minimal percentage of ingested organisms that were killed from the following equations: maximum viability index (MVI) = $\Sigma(a + b + c)/n_1$; phagocytic index (PI) = $d/n_2$; percent killed = $[(1 - \text{MVI/PI})] \times 100$. The values for $a$, $b$, $c$, and $n_1$ come from the malachite green–Diff-Quik spore stain results. Specifically, a is the total number of intracellular ungerminated spores, $b$ is the total number of intracellular germinated spores, $c$ is the total number of intracellular bacilli, and $n_1$ is the total number of macrophages examined. In the second equation, $d$ and $n_2$ come from the fluorescent "in/out" stain results. Specifically, $d$ is the total number of intracellular organisms counted and $n_2$ is the total number of macrophages. The calculation of the percent killed uses the PI and MVI to calculate the minimal percentage of ingested organisms killed inside the phagocyte.

The assay used to estimate the ability of RAW 264.7 murine macrophages to kill ingested *B. anthracis* spores is novel; however, its general concept resembles older staining procedures used to measure the abilities of neutrophils and monocytes to kill ingested bacteria and fungi (29, 30).

**Mouse challenge experiments.** Two inbred mouse strains (BALB/c and A/J) and one outbred strain (NMRI) were used. The BALB/c mice were challenged intraperitoneally or intranasally with potentially lethal doses of *B. anthracis* Ames spores. Treated groups received one or more doses of RC1 intraperitoneally or intranasally, and others served as untreated controls. NMRI mice were challenged subcutaneously with spores or intravenously with encapsulated *B. anthracis* Ames bacilli and treated with retrocyclins. A/J mice were challenged subcutaneously with *B. anthracis* Sterne spores and treated with retrocyclins intraperitoneally as described in detail below.

**Toxicity experiments.** Retrocyclin toxicity was studied in 20 A/J mice and 10 BALB/c mice. All mice were observed closely for signs of distress and were euthanized at selected time points. A/J mice received RC1 intraperitoneally with or without concomitant subcutaneous infection by *B. anthracis* Sterne. They were divided into 4 groups, each containing 5 mice. Group 1 (controls) received only intraperitoneal PBS. Group 2 received intraperitoneal PBS and subcutaneous *B. anthracis* Sterne spores. Group 3 received intraperitoneal RC1. Group 4 received intraperitoneal RC1 and subcutaneous *B. anthracis* Sterne spores. Mice were euthanized after 36, 48, 56, or 84 h.

The BALB/c mice received i.n. RC1 without a concomitant *B. anthracis* infection. Eight of these mice received 450 μg RC1 intranasally, and 2 mice received PBS by the same route. RC1-treated BALB/c mice were euthanized at 24, 36, and 48 h. Two RC1-treated BALB/c mice received a second dose of RC1 (~400 μg) after 48 h.

**Statistics.** Survival rates between groups were compared with Fisher exact tests. Kaplan-Meier analyses were used to construct survival curves and determine mean survival times, and survival curves were compared by log rank tests. Mean times to death were compared with *t* tests with permutation adjustment for multiple comparisons. The above analyses were done using SAS version 8.2 or the WEHI freeware (Melbourne, Australia).

**Research facilities.** The research was done in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and it adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals* (39a). The facilities wherein animal research was conducted are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal trials in Germany were approved under V201/04 according to the rules of the Animal Welfare Act. The *B. anthracis* experiments in Hohenheim, Germany, were conducted per the European normative prEN 13697:2000, with minor modifications.

## RESULTS

**Effects of serum.** Retrocyclins (50), as well as human α-defensins HNP1 to 3 (52) and HD5 (32), have lectin-like properties that allow them to bind serum glycoproteins such as fetuin, lactoferrin, IgA, and IgM reversibly but with high affinity. Because such binding may reduce the concentration of free RC available to interact with *B. anthracis* or its toxins, we examined the effects of serum on the ability of retrocyclins to kill *B. anthracis in vitro*. In one set of experiments, we incubated *B. anthracis* spores in 100% fetal calf serum for 90 min at 37°C, either in room air or in a 20% $CO_2$ atmosphere to obtain nonencapsulated and encapsulated germinated *B. anthracis*, respectively. Exposing such organisms to 0.5 to 1 μM RC1 for 2 h in a serum-free, citrate-glucose buffer caused a 3-$\log_{10}$ (~99.9%) fall in colony count, independent of capsule formation (Table 1). After germination and treatment in 100% human serum, 1 μM RC1 failed to kill any *B. anthracis*, but 10 μM RC1 killed 98.16% of encapsulated and 79.95% of nonencapsulated vegetative cells. The performance of RC1 in 100% mouse serum was even more impaired, as 10 μM RC1 failed to kill any *B. anthracis*; treatment with 100 μM RC1 killed >99% of both encapsulated and nonencapsulated vegetative cells. The inhibitory effects of serum on defensin-mediated activity against vegetative and spore forms of *B. anthracis* were also seen in our radial diffusion assays (Table 2), and HNP1 was especially susceptible to this effect. Table 2 also indicates that RC2 was approximately 3-fold more potent than RC1 in RPMI with or without 10% human or murine serum. Whereas PG1 and RC2 showed very similar potencies in serum-free underlay gels, the effectiveness of PG1 was not reduced when the underlay gels contained 10% human or murine serum with or without 50% RPMI tissue culture medium.

We used surface plasmon resonance (25) to determine the extent of serum binding by comparing the ability of human, murine, and fetal calf sera to inhibit the binding of retrocyclin to a biosensor displaying immobilized $gp120_{LAV}$ and/or gp41. Both glycoproteins are components of the major envelope glycoprotein of HIV-1 and are bound with high affinity by retrocyclins (20, 50). Table 3 shows that even serum concentrations of only 7.5% were sufficient to bind 75 to 90% of

TABLE 1. Effect of serum on susceptibility of germinated *B. anthracis* ΔAmes to RC1[a]

| Medium | RC1 concn (μM) | Encapsulated | | | Nonencapsulated | | |
|---|---|---|---|---|---|---|---|
| | | CFU ($10^5$) at $T_0$ | ΔCFU ($10^5$) at $T_1$ | Mean % killed | CFU ($10^5$) at $T_0$ | ΔCFU ($10^5$) at $T_1$ | Mean % killed |
| Buffer | 0.5 | 1.42 ± 0.23 | −1.41 ± 0.23 | 99.91 | 1.67 ± 0.25 | −1.66 ± 0.25 | 99.86 |
| | 1 | 1.42 ± 0.23 | −1.42 ± 0.23 | 99.93 | 1.67 ± 0.25 | −1.66 ± 0.25 | 99.92 |
| 100% human serum | 1 | 1.56 ± 0.14 | +0.98 ± 0.30 | 63.1 | 1.99 ± 0.07 | +0.57 ± 0.80 | 28.5 |
| | 10 | 2.28 ± 0.25 | −2.28 ± 0.26 | 98.16 | 1.62 ± 0.47 | −1.30 ± 0.44 | 79.95 |
| | 100 | 1.56 ± 0.14 | −1.56 ± 0.15 | 99.96 | 1.99 ± 0.07 | −1.99 ± 0.07 | 99.97 |
| 100% mouse serum | 1 | 2.16 ± 0.34 | +4.60 ± 1.96 | 212.6 | 1.77 ± 0.02 | +6.47 ± 2.41 | 364.9 |
| | 10 | 2.16 ± 0.34 | +6.40 ± 2.46 | 295.8 | 1.77 ± 0.02 | +6.84 ± 2.23 | 385.9 |
| | 100 | 2.16 ± 0.34 | −2.14 ± 0.34 | 99.10 | 1.77 ± 0.02 | −1.76 ± 0.02 | 99.08 |

[a] In these trials, $1 \times 10^5$ spores of ΔAmes were germinated in 0.1 ml serum for 90 min at 37°C, with or without 20% $CO_2$, and treated either in citrate buffer with glucose or with the indicated serum. All studies were performed with a $pXO1^-$ $pXO2^+$ strain, which formed capsules only when the spores were preincubated with $CO_2$. Data represent means ± standard deviations of three independent trials. Abbreviations: $T_0$, time when the germinated spore suspension was initially exposed to RC1; $T_1$, time at the end of the 2-h treatment with RC1; ΔCFU, change in CFU between $T_0$ and $T_1$. A plus sign signifies an increase in CFU, and a minus sign signifies a decrease.

retrocyclins 1 and 2 and that binding was substantially greater if the serum was subjected to conventional heat inactivation at 56°C for 30 min. The magnitude of this effect for native (i.e., not heat-inactivated) fetal calf serum is shown in Fig. 1.

We also used a two-stage kinetic assay (16) to examine the effects of native and heat-inactivated fetal calf, murine, and human serum on defensin-mediated activity against vegetative-phase *B. anthracis* Sterne. Figure 2a shows representative growth curves for *Bacillus anthracis*, and Fig. 2b shows the least mean squares regression fit of these growth curve data. Similar studies were done in every experiment to provide a standard curve and its equation, which were used to calculate bacterial survival, as described previously for other bacteria (16).

Panels a to c of Fig. 3 show the results of kinetic virtual colony count assays to determine the effects of 37.5% serum on the activity of human α-defensin HNP1 against *B. anthracis* Sterne. In serum-free DMEM, HNP1 was highly (>99.9%) effective at a concentration of 3.12 μg/ml. Native mouse and fetal calf sera each impaired the efficacy of HNP1 by about 15- to 30-fold. HI fetal calf and HI mouse sera were more inhibitory than the native sera. Native human serum was the least inhibitory of the three, as potency was impaired only 4- to 8-fold relative to serum-free DMEM. Panels d to f of Fig. 3 show the effects of 37.5% serum on the activities of RC1 and RC2. Although heat-inactivated mouse serum (Fig. 3f) was extremely inhibitory to both retrocyclins, native mouse, human, and fetal calf sera were not.

Figure 4 shows the activities of RC1 and RC2 against vegetative-phase *B. anthracis* in DMEM containing various concentrations of native fetal calf, mouse, or human serum. As the serum concentration increased, higher concentrations of retrocyclin were needed to obtain an equivalent effect. However, even in the presence of 75% human serum, 12.5 μg/ml (~6.5 μM) RC1 caused a 2-log decrease in calculated survival and RC2 caused a 3-log decrease. On a weight basis, both RC1 and RC2 were more effective than the human α-defensin HNP1.

Having gained some insights into the effects of serum on retrocyclin-mediated activity against vegetative-phase *B. an-*

TABLE 2. Two-stage radial diffusion assay results with gels impregnated with spore or vegetative forms of *B. anthracis* (Sterne)[a]

| *B. anthracis* Sterne form | Underlay addition(s) | MEC | | | | *n* |
|---|---|---|---|---|---|---|
| | | RC1 | RC2 | PG1 | HNP1 | |
| Spores* | None | 2.37 ± 0.06 | 0.81 ± 0.01 | 0.83 ± 0.05 | 25.8 ± 0.27 | 3 |
| | RPMI (R) | 2.18 ± 0.05 (d) | 0.72 ± 0.03 (d) | 0.81 ± 0.04 (d) | 24.2 ± 0.09 (b) | 3 |
| | Serum (mouse) | 7.22 ± 0.08 (c) | 2.47 ± 0.08 (c) | 0.28 ± 0.02 (c) | >250 (c) | 3 |
| | Serum (human) | 6.49 ± 0.07 (c) | 2.63 ± 0.03 (c) | 0.66 ± 0.04 (d) | >79.1, <250 (c) | 3 |
| | R + serum (mouse) | 8.58 ± 0.24 (c) | 2.72 ± 0.11 (c) | 0.25 ± 0.03 (c) | >250 (c) | 3 |
| | R + serum (human) | 7.18 ± 0.49 (c) | 2.75 ± 0.09 (c) | 0.78 ± 0.07 (d) | >79.1, <250 (c) | 3 |
| Vegetative cells | None | 2.27 ± 0.03 | 0.86 ± 0.02 | 0.99 ± 0.09 | 7.52 ± 0.12 | 3 |
| | RPMI (R) | 0.88 ± 0.03 (c) | 0.45 ± 0.11 (b) | 1.12 ± 0.05 (d) | 7.50 ± 0.03 (d) | 3 |
| | Serum (mouse) | 8.54 ± 0.23 (c) | 7.07 ± 0.31 (c) | 1.10 ± 0.06 (d) | 23.7 ± 0.23 (c) | 3 |
| | Serum (human) | 10.6 ± 0.61 (c) | 8.28 ± 0.37 (c) | 2.89 ± 0.13 (c) | 8.92 ± 0.43 (b) | 3 |
| | R + serum (mouse) | 6.88 ± 0.21 (c) | 2.14 ± 0.12 (c) | 0.68 ± 0.03 (b) | >79.1, <250 (c) | 3 |
| | R + serum (human) | 2.97 ± 0.17 (b) | 2.04 ± 0.00 (c) | 0.85 ± 0.00 (d) | 25.4 ± 0.00 (c) | 2 |

[a] All underlay gels had a volume of 10 ml and contained 10 mM sodium phosphate buffer, pH 7.4, 100 mM NaCl, 2% agarose, a 1:50 dilution of conventional tryptic soy broth, and approximately $5 \times 10^6$ viable spores or vegetative cells of *B. anthracis* Sterne. In addition, some underlay gels contained half-strength RPMI medium (R) and/or 10% (vol/vol) native mouse or human serum. The minimal effective concentration (MEC) values are means ± SEM, in μg/ml. *n* signifies the number of experiments. Letters in parentheses following data denote statistical significance of differences between MEC values obtained in assays with underlay gels that contained serum and/or RPMI relative to those from assays with underlay gels without these additions: b, $P < 0.01$; c, $P < 0.001$; d, $P > 0.05$. *, viable spores in the underlay that may have germinated during the 3-h period and allowed for the peptides to diffuse through the underlay gel, and they most certainly germinated during the overnight incubation that followed addition of the nutrient-rich top agar.

TABLE 3. Binding of retrocyclins by native and heat-inactivated sera[a]

| Serum source | Serum state | % free | |
|---|---|---|---|
| | | RC1 | RC2 |
| Fetal calf | Native | 17.6 ± 1.05 | 25.5 ± 4.0 |
| Mouse | Native | 25.7 ± 2.74 | 27.2 ± 3.5 |
| Human | Native | 19.4 ± 0.47 | 9.47 ± 3.2 |
| Fetal calf | Heated | 4.68 ± 0.96 | 4.28 ± 0.92 |
| Mouse | Heated | 14.9 ± 3.06 | 1.14 ± 0.67 |
| Human | Heated | 2.67 ± 0.90 | 0.65 ± 0.48 |

[a] The final serum concentration was 7.5%. RC1 and RC2 were at concentrations of 2.5, 5, 7.5, and 10 μg/ml. The values shown are means ± SEM for combined data from all four concentrations. Note that heat-inactivated sera bound considerably more RC1 and RC2 than did their native counterparts.

*thracis*, we next performed experiments to see if serum and/or red blood cells might affect the ability of RC1 to inhibit anthrax LF. In Fig. 5a and b, the assay mixtures contained LF (50 nM) from *Bacillus anthracis*, 20 mM HEPES (pH 7.4) with or without 25% (vol/vol) serum, with or without 25% washed mouse red blood cells, and with or without 50 μg/ml RC1. These components were preincubated at room temperature for 20 min. Then, the enzymatic reaction was started by adding substrate. Figure 5a shows that in serum-free buffer, the presence of red blood cells had no effect on the rate of lethal factor-mediated substrate hydrolysis. RC1 decreased lethal factor activity by 85.7% in the absence of red blood cells or serum, by 100% in the presence of red cells, and by ~15% in the presence of 25% mouse serum (Fig. 5b). "Careful" human serum (10 to 50% [vol/vol]), collected in a way that minimized contamination by constituents derived from leukocytes or platelets (41), had little effect on the enzymatic activity of lethal factor (Fig. 5c, curve B). However, whereas 50 μg/ml of RC1 inhibited lethal factor activity by over 90% in the absence of serum (Fig. 5c, curve D), its inhibition of lethal factor dropped to ~50% when 10% of careful human serum was present (Fig. 5c, curve C).

**Binding of RC1 to *B. anthracis* Sterne spores.** In three replicate experiments, *B. anthracis* spores in DMEM with or without 7.5% heat-inactivated fetal calf serum were exposed to 5 μg/ml of RC1. The concentration of RC1 that remained in the supernatant after binding was determined by surface plasmon resonance, using a gp120 biosensor (Fig. 1). In the absence of serum, the average number of molecules bound per spore was $2.43 \times 10^7$ in experiment 1, $2.29 \times 10^7$ in experiment 2, and $2.30 \times 10^7$ in experiment 3. When the binding study was performed in DMEM containing 7.5% heat-inactivated fetal calf serum, the number of RC1 molecules bound per spore was reduced by almost 80%, to $4.67 \times 10^6$ in experiment 1, $5.18 \times 10^6$ in experiment 2, and $5.79 \times 10^6$ in experiment 3.

**Subcutaneous challenge.** When A/J mice were infected subcutaneously with $5 \times 10^5$ *B. anthracis* Sterne spores, all (5/5) untreated control mice died from infection by day 4, but all 8 mice that received five intraperitoneal doses of RC1 (500-μg/dose at 12-h intervals) survived (Fig. 6a) ($P < 0.001$). Mice that received six 500-μg doses of RC1 at 24-h intervals fared less well (1 survivor of 5), although their survival was prolonged relative to untreated controls ($P < 0.005$). No deaths occurred in this group until after treatment with RC1 stopped.

**Intravenous challenge with encapsulated *B. anthracis* Ames.** The therapeutic efficacy of RC1 was also tested in a preliminary experiment with outbred NMRI mice, using the fully virulent Ames strain. The mice were challenged intravenously with $1 \times 10^3$ vegetative *B. anthracis* cells, which had been germinated in fetal calf serum and encapsulated by incubating them in 20% $CO_2$ at 37°C for 2 h. Although RC1 treatment (500 μg intraperitoneally) began immediately after infection and was repeated 6 h later, all of the mice died within 20 h after the second treatment, showing that this retrocyclin regimen was not effective in bacteremic infection with encapsulated *B. anthracis* (data not shown).

**Intraperitoneal challenge.** Female BALB/c mice, 6 to 12 weeks of age, were infected with *B. anthracis* Ames spores via the intraperitoneal route (Fig. 6b). Control group mice were not treated, while the other mice received three intraperitoneal 500-μg doses of RC1 or RC2. The first retrocyclin dose was given immediately after, but separately from, the *B. anthracis* challenge, and the remaining doses were given 8 and 24 h later. All 10 untreated controls were dead by day 3, but all 10 mice treated with RC1 survived. RC2 was less effective, as only 7 of the 10 treated mice survived infection.

**Intranasal challenge.** Mice received a pulmonary exposure to *B. anthracis* by intranasal inoculation with a spore suspension. The intranasal route was used, since the results with challenges by this route are comparable to those observed after aerosol exposure (6, 9). Figure 7a shows an experiment in which BALB/c mice received RC1 and *B. anthracis* Ames spores intranasally. When the peptide and spores were given together to ensure their colocalization in the lung, RC1 afforded complete protection ($P = 0.0007$ versus control). In contrast, when RC1 was given by the intranasal and intraperitoneal routes, respectively, after and separately from the spore challenge, no protection ensued. Thus, both the timing and the route of RC1 administration were of importance.

We also tested the prophylactic efficacy of RC1 by pretreat-

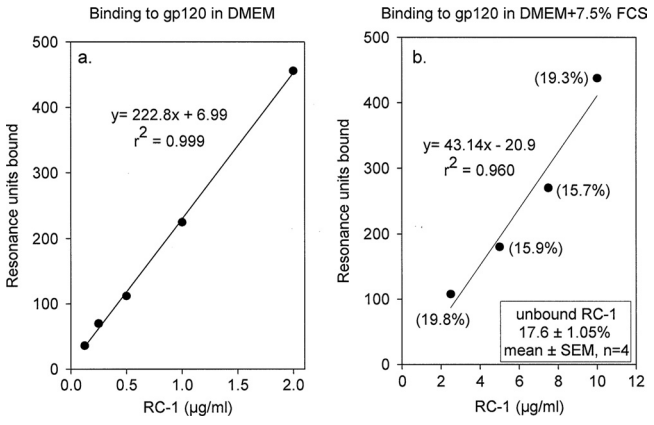


FIG. 1. Effect of FCS on availability of RC-1. Various concentrations of RC1 were delivered to the biosensor in either DMEM (a) or in DMEM plus 7.5% native fetal calf serum (b). Binding data (solid circles) were fit to standard curves by least mean squares linear regression lines to obtain the formulas that are shown. In panel b, numbers in parentheses indicate the percentage of total RC-1 that remained free, i.e., available to bind the biosensor. On average, in the presence of 7.5% FCS approximately 17.6% of the RC-1 was free and 82.4% was bound to FCS.

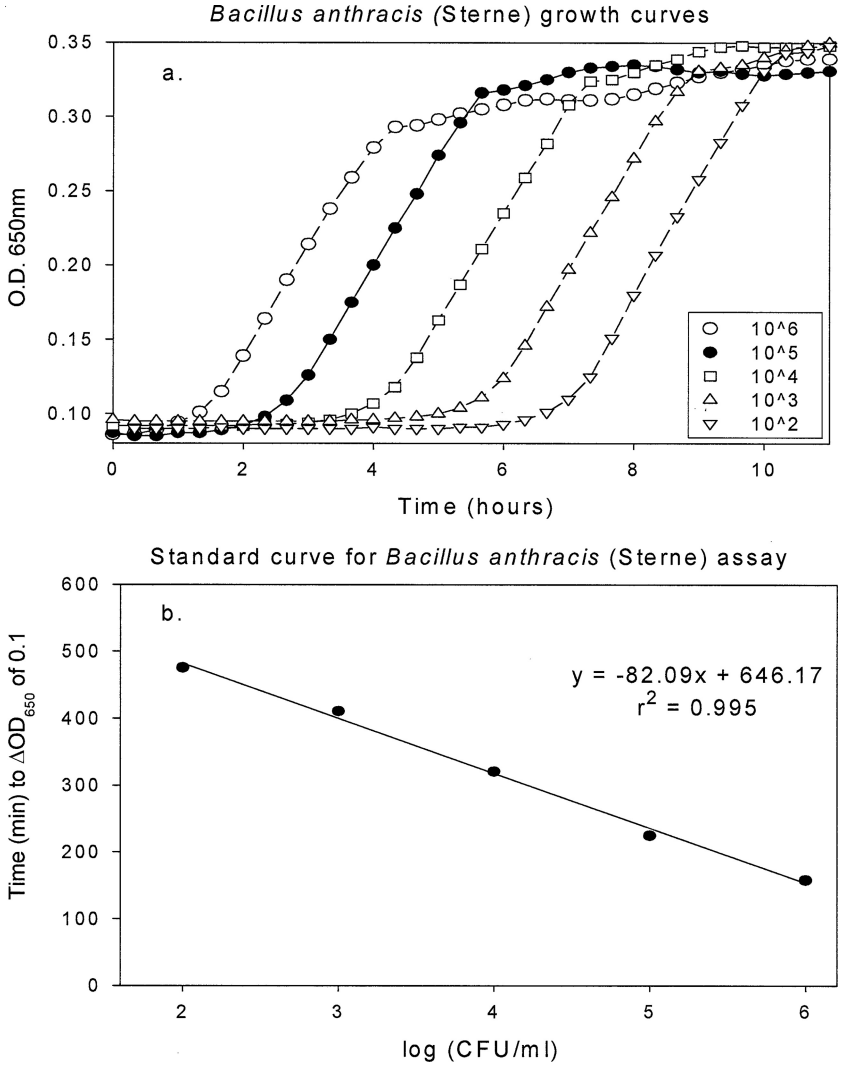


FIG. 2. Determination of a standard curve for the virtual colony count assay. (a) Growth in wells inoculated with 10-fold dilutions of vegetative *B. anthracis* cells that ranged from $10^2$ to $10^6$ CFU/ml. (b) Relationship between the $\log_{10}$ of the initial CFU/ml and the time, in minutes, for the optical density to increase by 0.1 absorbance units. The line, whose equation is shown, was fit by a least mean squares regression. These data are from a representative experiment, but similar growth curves and calculations were done in all experiments.

ing BALB/c mice with RC1 intranasally before challenging them with *B. anthracis* Ames spores via the intranasal route. Figure 7b shows the results of an experiment in which the mice received one 400-μg dose of RC1 intranasally 12 h before, 48 h before, 12 and 48 h before, or simultaneously with an intranasal challenge with $3.7 \times 10^5$ spores. The delay in time to death for mice given RC1 with or 12 h before the spore challenge was significant compared to that of the untreated controls. Three of 5 mice that received two 400-μg/kg intranasal doses of RC1 died prior to spore challenge, showing the toxicity of this regimen.

Given the inhibitory effects of serum on RC1 antimicrobial and antitoxic properties, the *in vivo* efficacy of RC1 was somewhat paradoxical, since it seemed unlikely to work effectively in the circulating blood. We wondered therefore if RC1 might be active in some other way. Our conjectures were guided by recent observations showing uptake of α-defensins by macrophages (44), epithelial cells (24), T lymphocytes (58), and endothelial and smooth muscle cells (4) and by indications that α-defensin uptake enhanced the phagocytic (17) or microbicidal (44) properties of macrophages.

**Association of retrocyclin with alveolar macrophages.** To determine if macrophages could acquire retrocyclins, we gave BALB/c mice fluorescently labeled RC by intranasal instillation, and approximately 12 h later we performed bronchoalveolar lavage to recover alveolar macrophages for flow cytometry studies. As we were unable to detect retrocyclin in the recovered alveolar macrophages, we obtained freshly lavaged alveolar macrophages from normal, uninfected BALB/c mice. After allowing these macrophages to become adherent, fluorescent retrocyclin was added and the incubation was continued for 3 h at 37°C in 5% $CO_2$. When these macrophages were examined by fluorescence microscopy, many were associated with the fluorescent construct (Fig. 8).

**Effects of RC1 on phagocytosis and killing of *B. anthracis* by RAW 264.7 cells.** Having shown that alveolar macrophages can

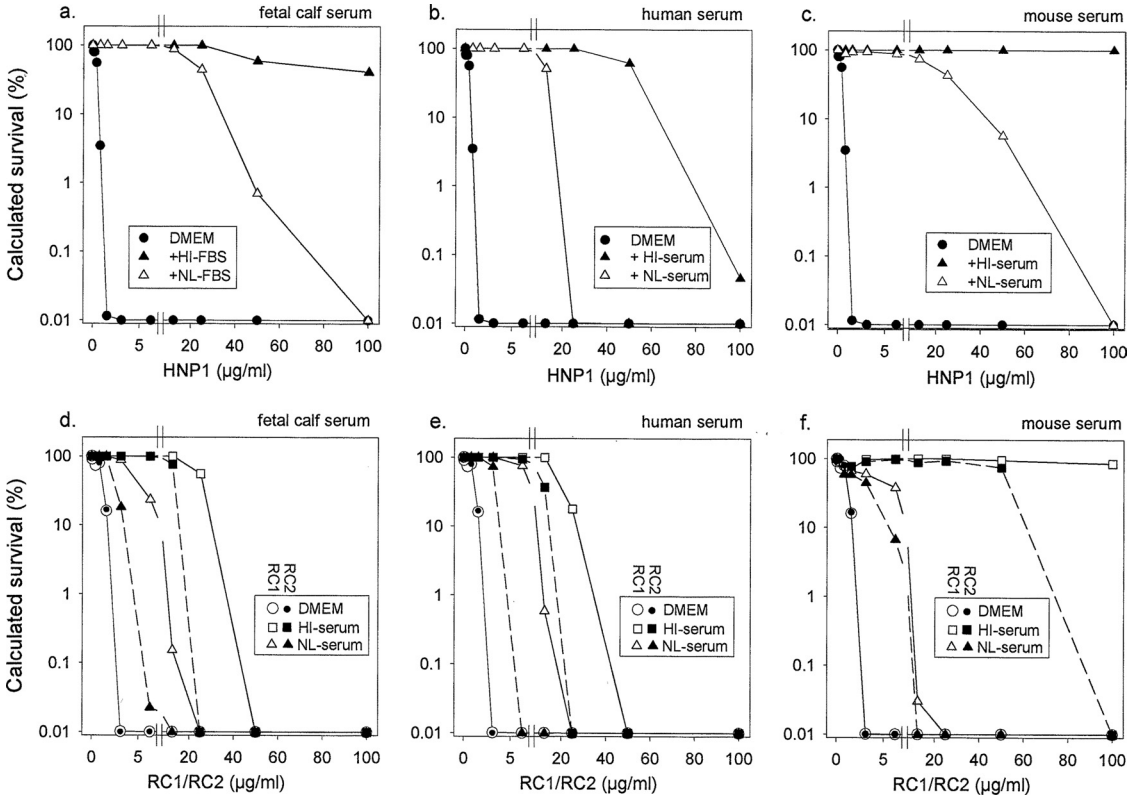


FIG. 3. Effects of native and heat-inactivated serum on the activity of HNP1 and RC1 against vegetative *B. anthracis* Sterne. Various concentrations of HNP1 (a to c) or RC1 and RC2 (d to f) were tested in DMEM with or without 37.5% (vol/vol) native or HI serum from fetal calf (a and d), normal human (b and e), or normal mouse (c and f). The symbols are defined in each panel.

acquire an exogenous retrocyclin construct, we next attempted to determine if RC1 uptake might enhance their ability to inhibit or kill ingested *B. anthracis* spores. In these experiments, *B. anthracis* spores that had been preincubated for 30 min with 0, 5, or 50 μg/ml of RC1 at 0°C were added to RAW 264.7 cells. These cultures were mixed, centrifuged for 10 min at 1,150 rpm (300 × *g*) at 30°C, and then incubated for 50 additional min at 37°C. At this point, the cocultures were washed extensively and incubated for an additional 30 min with medium containing gentamicin to kill germinated extracellular organisms and cytochalasin D to block further ingestion. The infected cultures were again washed extensively and either processed for viable counts or fixed and subjected to the immunofluorescence "in/out" or malachite green–Dif-Quik staining procedures.

Table 4 shows the percentage of ingested *B. anthracis* spores killed by the RAW 264.7 cells. This value was calculated from the PI and VI values, also shown in the table, using the equations described above. Whereas these macrophage-like cells killed only 18.0 ± 7.9% of untreated *B. anthracis* spores, they killed 63.0 ± 9.2% of spores that were preincubated with 5 μg/ml of RC1 ($P < 0.01$; $n = 6$ experiments). When the spores were preincubated with 50 μg/ml of RC1, an even higher percentage was killed (85.9 ± 3.34%; $n = 5$). The difference in killing of spores treated with 5 or 50 μg RC1/ml was statistically significant ($P = 0.012$ by paired $t$ test; $n = 5$).

Table 4 also shows the phagocytic index data. There was a trend toward increased uptake after RC1 pretreatment; however, the differences from the control were not statistically significant at either 5 or 50 μg/ml. The table also shows the viability index data. Here, the differences between RC1-treated and control spores were statistically significant at both 5 μg and 50 μg/ml. The difference between spores treated with 5 or 50 μg RC1/ml had a $P$ value of 0.020 (paired $t$ test; $n = 5$). We conclude that exposing *B. anthracis* spores to RC1 before they are ingested by murine RAW 264.7 macrophages results in significantly enhanced killing and that it may also increase their susceptibility to phagocytosis. The loss in viability was supported by colony count data acquired in four dilution plating experiments with RAW 264.7 cells exposed to untreated and retrocyclin-pretreated *B. anthracis* spores. Although the percentages of spore killing that was associated with RC1 treatment were lower as determined by viable counts than by staining, the relative CFU values were consistent with those shown in Table 4 (data not shown).

**Toxicity and pathology.** NMRI mice receiving intraperitoneal RC1 showed transient signs of distress (i.e., ruffled fur and hunching) that were similar to those observed for A/J mice, and these signs generally disappeared within a few hours. NMRI mice that were treated with RC1 after subcutaneous infection with *B. anthracis* Ames spores and ultimately died from the infection showed stronger edema than was noted in NMRI mice infected with Ames spores but not treated with RC1. NMRI mice not infected with *B. anthracis* but just treated with RC1 exhibited no edema. However, greater levels of edema were not noted in A/J mice infected with the Sterne strain and treated with RC1. In fact the edema was observed to be less than in control mice infected

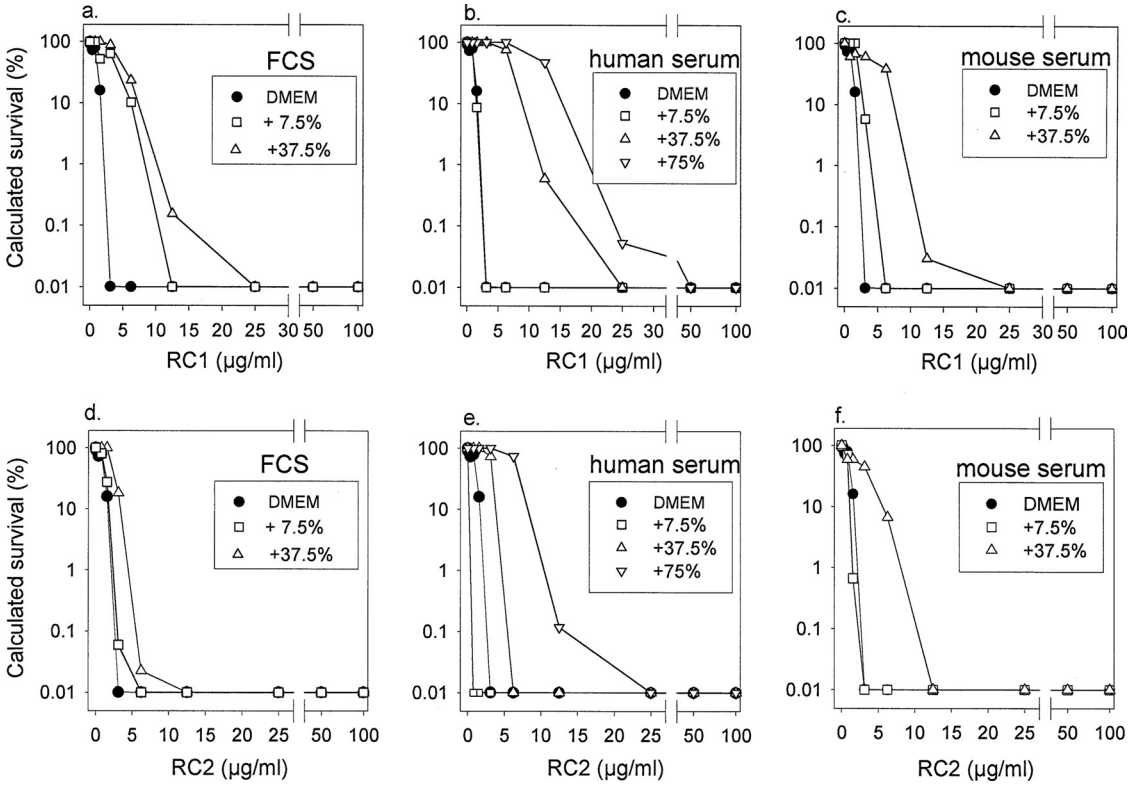


FIG. 4. Effects of native human serum on the activity of defensins against vegetative *B. anthracis* Sterne. Note that RC2 (open triangles) remains more potent than RC1 (open squares), and that both are more effective than HNP1 (solid circles) on a weight basis.

with Sterne spores but not treated with RC1. The BALB/c control mice and the A/J mice that received intraperitoneal RC1 with or without *B. anthracis* Sterne spores lacked identifiable microscopic pathology. The BALB/c mice given RC1 intranasally showed necrotizing bronchopneumonia and necrotizing tracheitis (Fig. 9 and data not shown).

## DISCUSSION

To date, translational studies on retrocyclins have centered on creating topical microbicides to prevent infection by HIV-1 and herpes simplex viruses (7, 19, 57). This is the first study showing that retrocyclins are protective *in vivo* against a seri-

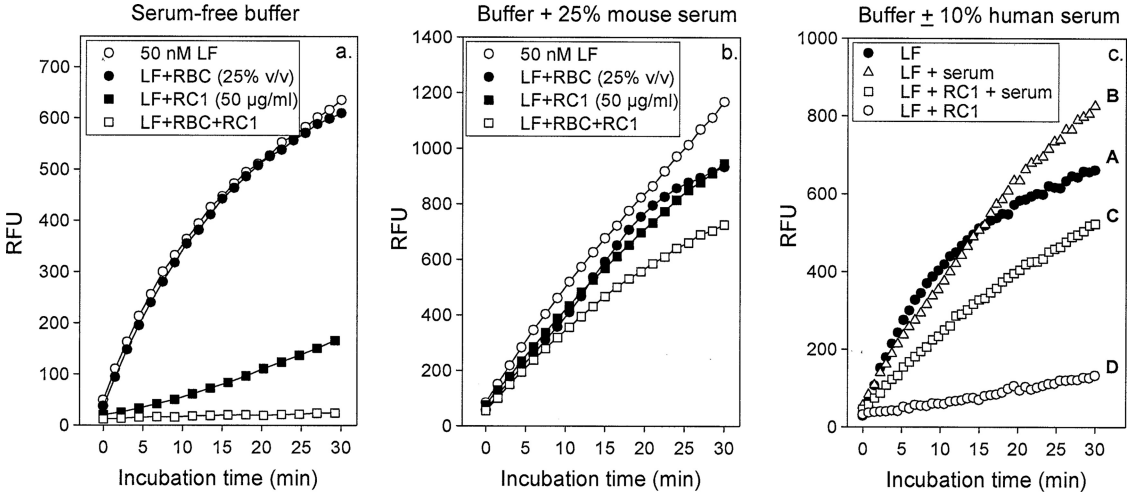


FIG. 5. Effects of serum and red blood cells (RBC) on inhibition of anthrax LF. Assays were performed in serum-free buffer (a), in buffer containing 25% mouse serum (b), or in buffer with or without 10% careful human serum (c). When present, washed mouse RBC at 25% (vol/vol) and anthrax LF at a final concentration of 50 nM were used. Assay components except for substrate were mixed together, preincubated for 20 min at room temperature, and then centrifuged for 60 s. After adding the resulting supernatants (80 μl) to microplate wells, substrate (20 μl) was added, and fluorescence was monitored for 30 min.

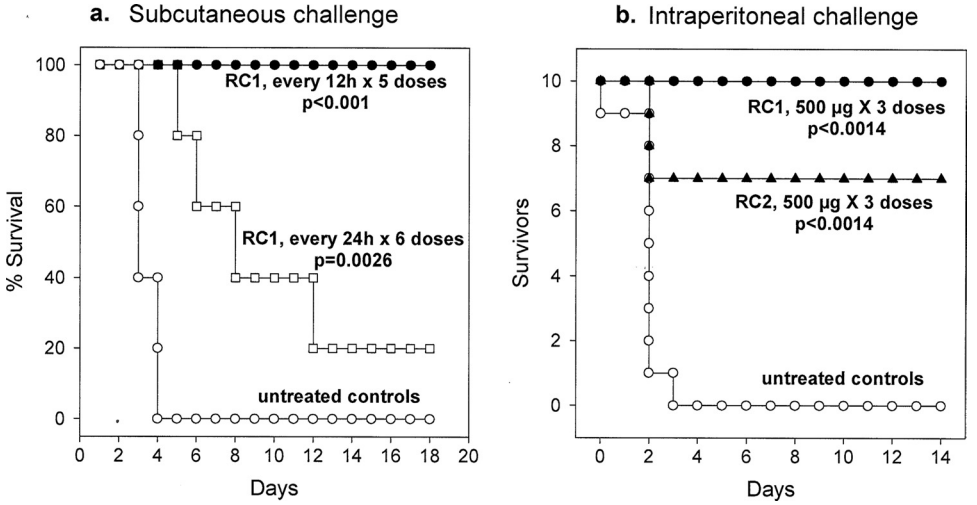


FIG. 6. Results after subcutaneous and intraperitoneal challenge. (a) A/J mice were infected subcutaneously with $5 \times 10^5$ *B. anthracis* Sterne spores. All untreated control mice (5/5) died from infection by day 4, but all 8 mice that received five intraperitoneal doses of RC1 (500-μg/dose) at 12-h intervals survived ($P < 0.001$). Mice given six 500-μg doses of RC1 at 24-h intervals did less well (1 survivor out of 5), although their survival was also prolonged relative to untreated controls ($P < 0.005$). No deaths occurred in this group until after treatment with RC1 stopped. (b) BALB/c mice were infected with approximately 2,000 *B. anthracis* Ames spores by intraperitoneal injection. The controls were untreated, and the other mice received three intraperitoneal doses, 500 μg each, of RC1 or RC2. The first retrocyclin dose was given immediately after, but separately from, the *B. anthracis* spores. The remaining RC doses were given 8 and 24 h later. Whereas all untreated controls (10/10) were dead by day 3, all (10/10) of the mice treated with RC1 survived for the duration of the experiment (14 days). RC2 was less effective, as only 7 of the 10 treated mice survived infection.

ous systemic bacterial pathogen. Retrocyclins 1 and/or 2 protected mice from *B. anthracis* spores given by three routes: subcutaneous, intraperitoneal, and intranasal. As formal pharmacokinetic studies were not done, dosing was based on maximally tolerated single intravenous doses in BALB/c mice. However, the ability of intraperitoneal retrocyclin to protect against subcutaneous *B. anthracis* spores implies that these peptides entered the circulation and were delivered to the infected tissue sites in therapeutically effective concentrations. Since retrocyclins may operate, in part, by enhancing macrophage performance, it would be informative to explore lower doses and shorter dosing intervals in future studies.

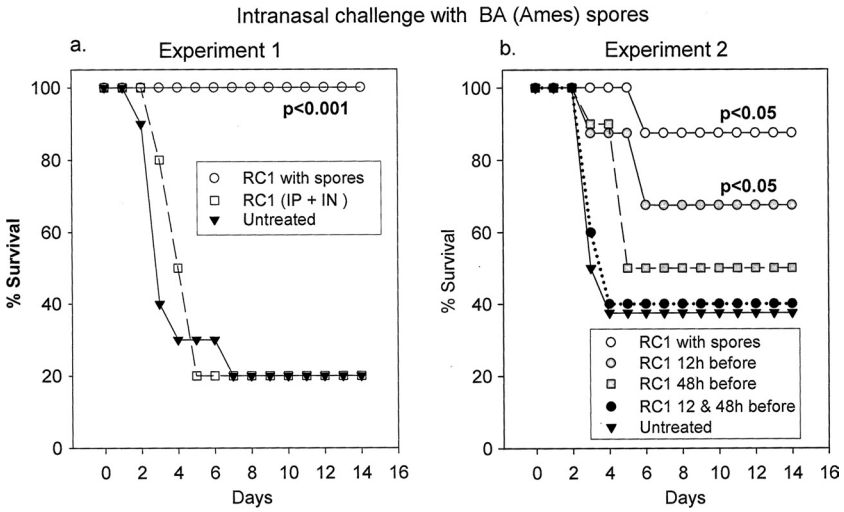


FIG. 7. Intranasal challenge results. (a) In experiment 1, $3.7 \times 10^5$ *B. anthracis* Ames spores were administered to BALB/c mice via the intranasal route. When RC1 (300 μg) and the spores were given together to ensure their colocalization in the lung, complete protection ensued ($P = 0.0007$ versus control). In contrast, when RC1 was given by the i.n. and intraperitoneal routes after and separately from the spore challenge (open squares), no protection was seen. The group receiving intraperitoneal and intranasal treatment received 500 μg of RC1 intraperitoneally immediately after the spores and 300 μg of RC1 intranasally 16 h after spore challenge. Untreated controls (solid inverted triangles) received spores but no RC1. (b) In experiment 2, BALB/c mice were challenged intranasally with approximately $1 \times 10^6$ *B. anthracis* Ames spores. A single intranasal administration of 400 μg of RC1 given either 12 h before the spore challenge or given along with the spores provided significant protection ($P < 0.05$). Delivering RC1 (400 μg) intranasally twice, 12 h and 48 h before the spore challenge (solid circles), did not protect the animals.

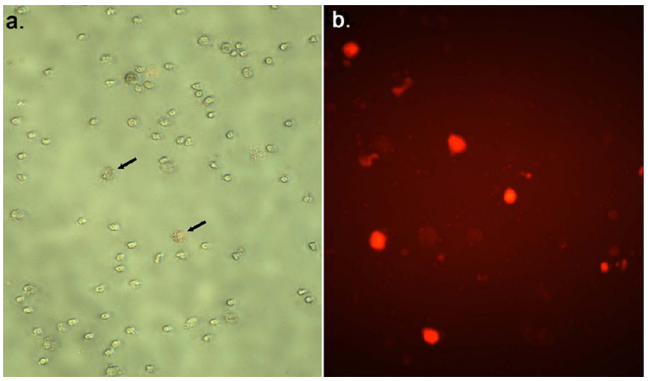


FIG. 8. Murine alveolar macrophages acquire exogenous retrocyclins. Alveolar macrophages, obtained by lavaging the lungs of normal BALB/c mice, were incubated for 3 h at 37°C in 5% $CO_2$ with 20 μg/ml of retrocyclin that had been covalently coupled to pegylated Alexa Fluor 568. (a) Photographed under phase microscopy; (b) photographed under fluorescence microscopy. Some macrophages appeared to have acquired enough retrocyclin to have reddish cytoplasm, even under phase microscropy (arrows, panel a).

Retrocyclins were fully protective or prolonged the survival time in A/J mice after subcutaneous infection with *B. anthracis* Sterne spores. They also protected mice from an intraperitoneal challenge with *B. anthracis* strain Ames spores when given intraperitoneally immediately after, but separately from, the intraperitoneal *B. anthracis* spore challenge. Protection was not observed for outbred NMRI mice challenged intraperitoneally with encapsulated *B. anthracis* Ames bacilli. These findings are consistent with an indirect, macrophage-dependent mechanism, since encapsulated *B. anthracis* Ames bacteria are less likely to be ingested than their nonencapsulated Sterne counterparts.

Exactly how retrocyclins are protective *in vivo* is not yet clear. The enhanced destruction of retrocyclin-treated *B. anthracis* spores following their ingestion by murine RAW 264.7 macrophages suggests that an important locus of retrocyclin-mediated protection may be intracellular. The same conclusion was reached in recent studies showing that human α-defensin HNP-1 and retrocyclin 1 enhanced the ability of RAW 264.7 macrophages to restrict vacuolar escape and intracellular pro-

TABLE 4. Effects of RC1 treatment on phagocytosis and killing[a]

| Expt no. and treatment | % of ingested spores killed | PI | VI |
|---|---|---|---|
| 1 | | | |
| Control | 10.7 | 0.384 | 0.343 |
| 5 μg/ml | 91 | 0.510 | 0.046 |
| 50 μg/ml | NT | NT | NT |
| 2 | | | |
| Control | 51.4 | 0.440 | 0.214 |
| 5 μg/ml | 57.9 | 0.580 | 0.244 |
| 50 μg/ml | 82.2 | 0.466 | 0.083 |
| 3 | | | |
| Control | 24.4 | 0.409 | 0.309 |
| 5 μg/ml | 55.8 | 0.495 | 0.219 |
| 50 μg/ml | 89.3 | 0.767 | 0.082 |
| 4 | | | |
| Control | 0.0 | 0.397 | 0.410 |
| 5 μg/ml | 42.0 | 0.274 | 0.159 |
| 50 μg/ml | 76.1 | 0.381 | 0.091 |
| 5 | | | |
| Control | 21.5 | 0.275 | 0.216 |
| 5 μg/ml | 90.4 | 0.502 | 0.048 |
| 50 μg/ml | 96.0 | 0.605 | 0.024 |
| 6 | | | |
| Control | 0.0 | 0.338 | 0.620 |
| 5 μg/ml | 41.2 | 0.534 | 0.314 |
| 50 μg/ml | 85.9 | 0.823 | 0.116 |
| Mean ± SEM (*n*) | | | |
| Control | 18.0 ± 7.89 (6) | 0.374 ± 0.024 (6) | 0.352 ± 0.062 (6) |
| 5 μg/ml | 63.0 ± 9.18 (6) | 0.482 ± 0.044 (6) | 0.172 ± 0.044 (6) |
| 50 μg/ml | 85.9 ± 3.34 (5) | 0.608 ± 0.085 (5) | 0.079 ± 0.015 (5) |
| *P* value | | | |
| Control vs. treated | 0.009 | 0.085 | 0.020 |
| Control vs. 50 μg/ml | 0.002 | 0.074 | 0.013 |
| 5 μg/ml vs. 50 μg/ml | 0.012 | NA | 0.020 |

[a] The equations used to calculate the PI (phagocytic index), VI (viability index), and percentage of ingested spores killed are defined in Materials and Methods. NT, not tested: NA. not applicable. The differences between RC1-treated and control spores were statistically significant at both 5 and 50 μg/ml by paired *t* tests. The PI data suggest enhanced uptake of spores pretreated with RC1; however, the difference from the control was not statistically significant by paired *t* test.

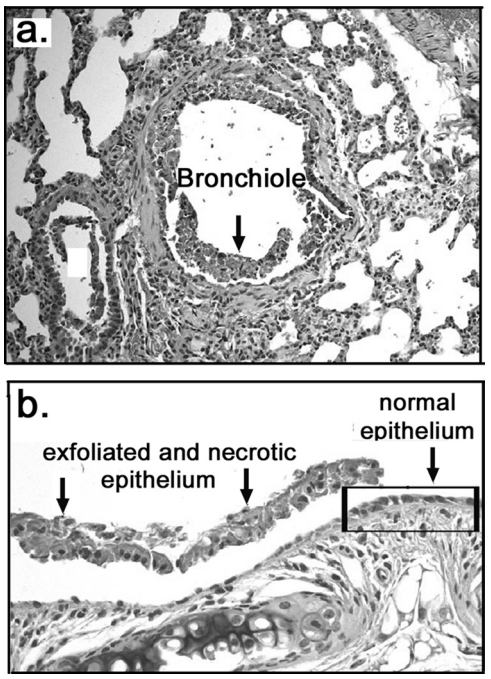


FIG. 9. Airway toxicity after intranasal RC1 administration. (a) Bronchioles containing exfoliated epithelium. (b) An area with intact tracheal epithelium (boxed) adjacent to areas showing tracheitis and epithelial exfoliation (arrows).

liferation by *Listeria monocytogenes* (2). These studies documented extensive HNP1 uptake by the macrophages and the appearance of HNP1 within phagosomes containing ingested bacteria.

Binding of retrocyclin to *B. anthracis* spores was linearly related to its concentration. *B. anthracis* spores exposed to 5 μg/ml of RC1 in Dulbecco's medium supplemented with 7.5% fetal calf serum bound, on average, $2.34 \times 10^7$ RC1 molecules/spore. In plasmon resonance experiments with native and heat-inactivated sera, 7.5% heat-inactivated fetal calf serum reduced the concentration of free (i.e., unbound) RC1 by 95.3% (Table 3). If only this unbound free RC1 could bind *B. anthracis* spores, we would expect about $1.10 \times 10^6$ RC1 molecules bound per spore [$4.68\% \times (2.34 \times 10^7)$] in such serum. However, RC1 binding in Dulbecco's medium containing 7.5% heat-inactivated fetal calf serum was considerably higher ([$5.21 \pm 0.32$] $\times 10^6$ RC1 molecules/spore), showing that RC1's affinity for *B. anthracis* spores exceeded its affinity for some of its binding proteins in heat-inactivated fetal calf serum.

Based on these binding data and the earlier findings of Sbarra et al. (42), we hypothesize that spore-bound RC1 molecules accompany ingested *B. anthracis* spores into phagosomes via "piggyback phagocytosis." Carrera et al. (5) calculated that the average volume of *B. anthracis* Sterne was $0.569 \pm 0.14$ $\mu m^3$ by treating them as ellipsoids whose volume ($V$) equaled $\pi LW^2/6$, where $L$ is length and $W$ is the diameter. If we add 100 nm (0.1 μm) to the spore's length and width to approximate its separation from the phagosomal membrane (14), we can use the formula to estimate that the total phagosomal volume would be 0.976 $\mu m^3$, with 58% of it occupied by the spore. Since 1 liter contains $10^{15}$ $\mu m^3$, the concentration of RC1 in the spore-containing phagosome corresponds to $5.34 \times 10^{21}$ molecules per liter. Applying Avogadro's number to convert this RC1 concentration to molarity gives a result 8.87 mM, which is rather startling, since it is almost 3,400-fold higher than the extracellular RC1 concentration (5 μg/ml; 2.61 μM). Since the extracellular binding of RC1 is noncovalent and potentially reversible, its behavior in the phagosomal compartment could differ considerably from its extracellular behavior. The possibility that piggyback phagocytosi allows macrophages to generate very high local concentrations of defensins or other neutrophil-derived molecules within their phagosomes is a novel concept that deserves further study.

The importance of phagocytes in host resistance to *B. anthracis* has strong historical (38) as well as recent support. Human PMN that ingested spores of wild-type, capsule-deficient, or toxin-deficient *B. anthracis* strains allowed these spores to germinate intracellularly and then killed them so efficiently that only 30% remained viable by 4 h. This intracellular killing was attributed to human α-defensins, rather than to PMN-derived reactive oxygen species (37). Whereas defensin-deficient mouse neutrophils, which are known to lack defensins (15), play a minor role in the early host response to *B. anthracis* spores, murine macrophages make substantial contributions (11, 13).

By design, the doses of retrocyclin used in these experiments verged on the toxic. Consequently, many treated mice showed signs of transient distress, and two uninfected mice who received the peptide intranasally died with evidence of damage to their tracheal epithelium. Yet, despite inducing pulmonary toxicity, RC1 protected against an otherwise-lethal spore challenge. In reporting the protective ability of rhesus θ-defensin 1 (RTD-1), a rhesus macaque θ-defensin, in a murine severe acute respiratory syndrome-like coronavirus model, investigators noted that intranasal RTD-1 produced dose-dependent changes in airway tissue histopathology and that doses of 2.4 mg/kg caused "significant lesions" 2 and 4 days after administration (56). The doses of intranasal RC1 used in our experiments were many times higher than that dose.

Intranasal administration does not allow precise dose delivery of RC1 to the airways and alveoli. Consequently, the two uninfected mice that died after their i.n. RC1 treatment may simply have internalized more peptide than the other mice. For RC1 given via the airways, its most important target is the very thin layer of epithelial lining fluid (ELF) on the air side of the alveolo-capillary interface. A human lung weighs about 1,200 g and contains about 375 million alveoli (1), whose surfaces are moistened by 15 to 20 ml of this ELF (36, 53). By simple extrapolation, a mouse lung weighing about 150 mg may contain less than 2.5 μl of ELF (46). Consequently, if only 1% of an intranasal dose of 300 or 400 μg RC1 reaches the ELF, its local RC1 concentration would exceed 1 mg/ml. Accordingly, much lower intranasal doses of retrocyclin and alternative means of delivering it to the airways would merit consideration in the future. Since retrocyclins and other θ-defensins are active both *in vivo* and *in vitro*, further development of these intriguing molecules is warranted.

## ACKNOWLEDGMENTS

Synthetic HNP1 was provided by Wuyuan Lu of the Department of Biochemistry and the Institute for Human Virology of the University of Maryland, Baltimore, MD. We thank Kenneth Bradley for providing *B. anthracis* lethal factor and *B. anthracis* Sterne spores and for allowing us to use his laboratory for experiments with this organism. We also thank Eugene Gillespie and Byran France from the Bradley lab for their assistance.

The USAMRIID research described in this paper was supported by JSTO-CBD/DTRA project 1.1A0010-07-RDB. The Universität Hohenheim research was funded by the German Ministry of Defense (grant E/UR3G/5G002/5A800). Research at UCLA was funded by a grant, RO1-AI070726, from the NIH.

The views expressed in this report are those of the authors and do not reflect official policy or position of the Department of the Army, Department of Defense or the U.S. Government.

## REFERENCES

1. **Angus, G. E., and W. M. Thurlbeck.** 1972. Number of alveoli in the human lung. J. Appl. Physiol. **32:**483–485.
2. **Arnett, E., R. I. Lehrer, P. Pratikhya, W. Lu, and S. Seveau.** 2011. Defensins enable macrophages to inhibit the intracellular proliferation of *Listeria monocytogenes*. Cell. Microbiol. **13:**635–651.
3. **Banks, D. J., M. Barnajian, F. J. Maldonado-Arocho, A. M. Sanchez, and K. A. Bradley.** 2005. Anthrax toxin receptor 2 mediates Bacillus anthracis killing of macrophages following spore challenge. Cell. Microbiol. **7:**1173–1185.
4. **Bdeir, K., et al.** 1999. Defensin promotes the binding of lipoprotein(a) to vascular matrix. Blood **94:**2007–2019.
5. **Carrera, M., R. O. Zandomeni, J. Fitzgibbon, and J. L. Sagripanti.** 2007. Difference between the spore sizes of *Bacillus anthracis* and other *Bacillus* species. J. Appl. Microbiol. **102:**303–312.
6. **Cleret, A., et al.** 2007. Lung dendritic cells rapidly mediate anthrax spore entry through the pulmonary route. J. Immunol. **178:**7994–8001.
7. **Cole, A. L., et al.** 2007. The retrocyclin analogue RC-101 prevents human immunodeficiency virus type 1 infection of a model human cervicovaginal tissue construct. Immunology **121:**140–145.
8. **Cole, A. M., et al.** 2002. Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. Proc. Natl. Acad. Sci. U. S. A. **99:**1813–1818.
9. **Cote, C. K., J. Bozue, N. Twenhafel, and S. L. Welkos.** 2009. Effects of altering the germination potential of *Bacillus anthracis* spores by exogenous means in a mouse model. J. Med. Microbiol. **58:**816–825.
10. **Cote, C. K., et al.** 2008. Early interactions between fully virulent Bacillus anthracis and macrophages that influence the balance between spore clearance and development of a lethal infection. Microbes Infect. **10:**613–619.
11. **Cote, C. K., K. M. Rea, S. L. Norris, N. van Rooijen, and S. L. Welkos.** 2004. The use of a model of in vivo macrophage depletion to study the role of macrophages during infection with Bacillus anthracis spores. Microb. Pathog. **37:**169–175.
12. **Cote, C. K., et al.** 2005. The detection of protective antigen (PA) associated with spores of *Bacillus anthracis* and the effects of anti-PA antibodies on spore germination and macrophage interactions. Microb. Pathog. **38:**209–225.
13. **Cote, C. K., N. van Rooijen, and S. L. Welkos.** 2006. Roles of macrophages and neutrophils in the early host response to Bacillus anthracis spores in a mouse model of infection. Infect. Immun. **74:**469–480.
14. **de Chastellier, C.** 2008. EM analysis of phagosomes. Methods Mol. Biol. **445:**261–285.
15. **Eisenhauer, P. B., and R. I. Lehrer.** 1992. Mouse neutrophils lack defensins. Infect. Immun. **60:**3446–3447.
16. **Ericksen, B., Z. Wu, W. Lu, and R. I. Lehrer.** 2005. Antibacterial activity and specificity of the six human α-defensins. Antimicrob. Agents Chemother. **49:**269–275.
17. **Fleischmann, J., M. E. Selsted, and R. I. Lehrer.** 1985. Opsonic activity of MCP-1 and MCP-2, cationic peptides from rabbit alveolar macrophages. Diagn. Microbiol. Infect. Dis. **3:**233–242.
18. **Friedlander, A. M., S. L. Welkos, and B. E. Ivins.** 2002. Anthrax vaccines. Curr. Top. Microbiol. Immunol. **271:**33–60.
19. **Fuhrman, C. A., et al.** 2007. Retrocyclin RC-101 overcomes cationic mutations on the heptad repeat 2 region of HIV-1 gp41. FEBS J. **274:**6477–6487.
20. **Gallo, S. A., et al.** 2006. Theta-defensins prevent HIV-1 Env-mediated fusion by binding gp41 and blocking 6-helix bundle formation. J. Biol. Chem. **281:**18787–18792.
21. **Ganz, T.** 2003. Defensins: antimicrobial peptides of innate immunity. Nat. Rev. Immunol. **3:**710–720.
22. **Garcia, A. E., G. Osapay, P. A. Tran, J. Yuan, and M. E. Selsted.** 2008. Isolation, synthesis, and antimicrobial activities of naturally occurring theta-defensin isoforms from baboon leukocytes. Infect. Immun. **76:**5883–5891.
23. **Hanna, P.** 1998. Anthrax pathogenesis and host response. Curr. Top. Microbiol. Immunol. **225:**13–35.
24. **Hazrati, E., et al.** 2006. Human alpha- and beta-defensins block multiple steps in herpes simplex virus infection. J. Immunol. **177:**8658–8666.
25. **Howard, M. L., J. J. Hill, G. R. Galluppi, and M. A. McLean.** 2010. Plasma protein binding in drug discovery and development. Comb. Chem. High Throughput Screen. **13:**170–187.
26. **Ivins, B. E., et al.** 1986. Immunization studies with attenuated strains of *Bacillus anthracis*. Infect. Immun. **52:**454–458.
27. **Kim, C., et al.** 2005. Human alpha-defensins neutralize anthrax lethal toxin and protect against its fatal consequences. Proc. Natl. Acad. Sci. U. S. A. **102:**4830–4835.
28. **Kokryakov, V. N., et al.** 1993. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. FEBS Lett. **327:**231–236.
29. **Lehrer, R. I.** 1970. Measurement of candidacidal activity of specific leukocyte types in mixed cell populations. I. Normal, myeloperoxidase-deficient, and chronic granulomatous disease neutrophils. Infect. Immun. **2:**42–47.
30. **Lehrer, R. I.** 1975. The fungicidal mechanisms of human monocytes. I. Evidence for myeloperoxidase-linked and myeloperoxidase-independent candidacidal mechanisms. J. Clin. Invest. **55:**338–346.
31. **Lehrer, R. I.** 2007. Multispecific myeloid defensins. Curr. Opin. Hematol. **14:**16–21.
32. **Lehrer, R. I., et al.** 2009. Multivalent binding of carbohydrates by the human alpha-defensin, HD5. J. Immunol. **183:**480–490.
33. **Lehrer, R. I., A. K. Lichtenstein, and T. Ganz.** 1993. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. Annu. Rev. Immunol. **11:**105–128.
34. **Lehrer, R. I., M. Rosenman, S. S. Harwig, R. Jackson, and P. Eisenhauer.** 1991. Ultrasensitive assays for endogenous antimicrobial polypeptides. J. Immunol. Methods **137:**167–173.
35. **Leighton, T. J., and R. H. Doi.** 1971. The stability of mRNA during sporulation in Bacillus subtilis. J. Biol. Chem. **246:**3189–3195.
36. **Macklin, C. C.** 1954. The pulmonary alveolar mucoid film and the pneumonocytes. Lancet **266:**1099–1104.
37. **Mayer-Scholl, A., et al.** 2005. Human neutrophils kill *Bacillus anthracis*. PLoS Pathog. **1:**e23.
38. **Metchnikoff, E.** 1905. Immunity to infective diseases. Cambridge University Press, Cambridge, United Kingdom.
39. **Moir, A., and D. A. Smith.** 1990. The genetics of bacterial spore germination. Annu. Rev. Microbiol. **44:**531–553.
39a. **National Research Council.** 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, DC.
40. **Nguyen, T. X., A. M. Cole, and R. I. Lehrer.** 2003. Evolution of primate theta-defensins: a serpentine path to a sweet tooth. Peptides **24:**1647–1654.
41. **Panyutich, A. V., N. N. Voitenok, R. I. Lehrer, and T. Ganz.** 1991. An enzyme immunoassay for human defensins. J. Immunol. Methods **141:**149–155.
42. **Sbarra, A. J., W. Shirley, and W. A. Bardawil.** 1962. 'Piggy-back' phagocytosis. Nature **194:**255–256.
43. **Steinberg, D. A., and R. I. Lehrer.** 1997. Designer assays for antimicrobial peptides. Disputing the "one-size-fits-all" theory. Methods Mol. Biol. **78:**169–186.
44. **Tan, B. H., et al.** 2006. Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens. J. Immunol. **177:**1864–1871.
45. **Tang, Y. Q., et al.** 1999. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. Science **286:**498–502.
46. **Thompson, D. C., and M. A. Trush.** 1988. Studies on the mechanism of enhancement of butylated hydroxytoluene-induced mouse lung toxicity by butylated hydroxyanisole. Toxicol. Appl. Pharmacol. **96:**122–131.
47. **Turnbull, P. C. B.** 2002. Introduction: anthrax history, disease and ecology, p. 1–20. *In* T. Koehler (ed.), Anthrax. Springer Verlag, Berlin, Germany.
48. **Turner, J., Y. Cho, N. N. Dinh, A. J. Waring, and R. I. Lehrer.** 1998. Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. Antimicrob. Agents Chemother. **42:**2206–2214.
49. **Venkataraman, N., et al.** 2009. Reawakening retrocyclins: ancestral human defensins active against HIV-1. PLoS Biol. **7:**e95.
50. **Wang, W., A. M. Cole, T. Hong, A. J. Waring, and R. I. Lehrer.** 2003. Retrocyclin, an antiretroviral theta-defensin, is a lectin. J. Immunol. **170:**4708–4716.
51. **Wang, W., et al.** 2006. Retrocyclins kill bacilli and germinating spores of *Bacillus anthracis* and inactivate anthrax lethal toxin. J. Biol. Chem. **281:**32755–32764.
52. **Wang, W., et al.** 2004. Activity of alpha- and theta-defensins against primary isolates of HIV-1. J. Immunol. **173:**515–520.
53. **Watford, W. T., A. J. Ghio, and J. R. Wright.** 2000. Complement-mediated host defense in the lung. Am. J. Physiol. Lung Cell. Mol. Physiol. **279:**L790–L798.
54. **Welkos, S., A. Friedlander, S. Weeks, S. Little, and I. Mendelson.** 2002. In-vitro characterisation of the phagocytosis and fate of anthrax spores in

macrophages and the effects of anti-PA antibody. J. Med. Microbiol. **51:**821–831.
55. **Welkos, S. L., R. W. Trotter, D. M. Becker, and G. O. Nelson.** 1989. Resistance to the Sterne strain of *B. anthracis*: phagocytic cell responses of resistant and susceptible mice. Microb. Pathog. **7:**15–35.
56. **Wohlford-Lenane, C. L., et al.** 2009. Rhesus theta-defensin prevents death in a mouse model of severe acute respiratory syndrome coronavirus pulmonary disease. J. Virol. **83:**11385–11390.
57. **Yasin, B., et al.** 2004. Theta defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. J. Virol. **78:**5147–5156.
58. **Zhang, L., et al.** 2002. Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. Science **298:**995–1000.